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Regulation of Dkk1, an Inhibitor of the Wnt Signaling Pathway, by DAX-1 in Mouse Embryonic Stem Cells

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By

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A thesis submitted for the Biology Honors Program at The University of San Francisco

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Abstract

DAX-1 is known to play a key role in the maintenance of pluripotency in mouse embryonic stem cells (mESC), such that elimination of DAX-1 (through methods such as RNAi) leads to the differentiation of ESC (Khalfallah et al. 2009). Earlier research by the Tzagarakis-Foster lab identified target genes of DAX-1 in mESC through PCR array methodology (Torres 2013). The Dickkopf 1 gene (Dkk1) was identified as a potential target of DAX-1 by these studies and has since been confirmed to be a DAX-1 target in mESC. However, to date very little is understood of the mechanism of DAX-1 repression of Dkk1 in mESC. In order to better understand the relationship between DAX-1 and Dkk1 in mESC, DAX-1 was knocked down via RNAi and levels of gene expression were measured by qPCR. We found that Dkk1 gene expression exhibited more than a twofold increase in mESCs that had a DAX-1 knockdown, which supports our hypothesis that DAX-1 acts as a negative transcriptional regulator of Dkk1.

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List of Abbreviations

AF-1/AF-2:	Autonomous transcriptional activation Function 1/2
APC:	Adenomatous Polypsis Coli
C:	Cysteine
cDNA:	Complementary DNA
CK1:	Casein Kinase 1
Cys:	Cysteine
DAX-1:	Dosage sensitive sex reversal, Adrenal hypoplasia congenita, critical region on the X chromosome, gene 1
DBD:	DNA Binding Domain
Dkk:	Dickkopf
DMEM:	Dulbecco's Modified Eagle Medium
DNA:	Deoxyribonucleic Acid
Dvl:	Dishevelled
ESC:	Embryonic Stem Cells
Fz:	Frizzled
G:	Glycine
GAPDH:	Glyceraldehyde 3-Phosphate Dehydrogenase
GSK-3β:	Glycogen Synthase Kinase 3β
H:	Histidine
K:	Lysine
L:	Leucine

LBD:	Ligand Binding Domain
LEF:	Lymphoid Enhancer Factor
LRP 5/6:	Low-Density Lipoprotein Related Receptor Protein 5/6
mESC:	mouse Embryonic Stem Cells
NHR:	Nuclear Hormone Receptor
P:	Proline
PBS:	Phosphate Buffered Saline
PCR:	Polymerase Chain Reaction
qPCR:	Quantitative Polymerase Chain Reaction
RNA:	Ribonucleic Acid
RNAi:	RNA interference
S:	Serine
Ser:	Serine
SF1:	Steroidogenic Factor 1
siRNA:	small interfering RNA
SOX9:	SRY-related HMG box9
SRY:	Sex-determining Region Y
TAE:	Tris-acetate EDTA
TCF:	TCell Factor
TDF:	Testes Determining Factor
Thr:	Threonine
X:	Any Amino Acid
Wnt:	Wingless/integration1

Introduction

Wnt Signaling

The Wnt (**w**ingless/**i**ntegration1) signaling pathway is a signal transduction cascade that is key mediator of both embryonic development from *Drosophila* to mammals as well as certain cancers (Wodarz and Nusse 1998; Logan and Nusse 2004). In embryonic development, the Wnt pathway has many roles, including anterior-posterior axial patterning, somitogenesis, angiogenesis, vasculogenesis, and limb, bone, tooth and eye formation (Logan and Nusse 2004; Katoh 2007). Wnt signaling is activated by the release of Wnt proteins, which can act in either an autocrine or paracrine fashion.

The Wnt proteins are a family of glycoproteins that are 350-400 amino acids long and have either 23 or 24 conserved cysteine residues. Additionally, all Wnt proteins share a common CKCHGXSGXC motif (Mikels and Nusse 2006). In mammals, 19 Wnt proteins have been identified. The diversity of the Wnt family of proteins is conserved across species from cnidarians (Hydra) to humans. However, to date they have not been found in prokaryotes, unicellular eukaryotes, or plants (Miller 2001). This suggests that Wnt signaling is necessary for the development of most multicellular organisms (Amerongen and Nusse 2009). Wnt proteins are generally split into two groups. The first group includes the canonical Wnt ligands. These Wnt proteins signal to stabilize β-catenin, which combines with the TCF/LEF (**TC**ell

Factor/**L**ymphoid **E**nhancer **F**actor) complex to change transcription of target genes (Kawano and Kypta 2003). The second group includes the non-canonical Wnt ligands. Wnt proteins in this group activate other signaling pathways, such as the Wnt/Ca^{2+} or Wnt/polarity pathway (Miller 2001).

The canonical Wnt signaling pathway is activated when secreted Wnt proteins bind to the seven transmembrane domain receptor Fz (**F**ri**z**zled) as well as LRP 5/6 (**L**ow-Density Lipoprotein Related **R**eceptor **P**rotein **5** or **6**). These three molecules form a complex that recruits the protein Dvl (**D**ishe**v**e**l**led) (MacDonald et al. 2009). Dvl then recruits Axin, a scaffold protein, to the complex, binding to its PPPSPXS motifs of LRP 5/6. Axin also recruits GSK-3β (**G**lycogen **S**ynthase **K**inase-**3β**) and CK1 (**C**asein **K**inase **1**), which in turn phosphorylate LRP 5/6 on all five of its PPPSPXS motifs. This phosphorylation is essential for signaling downstream in the pathway (Figure 1) (Tamai et al. 2004). Loss of Axin from the Axin complex leads to a failure of GSK-3β and CK1 to phosphorylate β-catenin and mark it for degradation. Free β-catenin is then allowed to accumulate in the cytosol and eventually enter the nucleus, where it works with other transcription factors, such as TCF/LEF, to regulate the transcription of target genes (MacDonald et al. 2009).

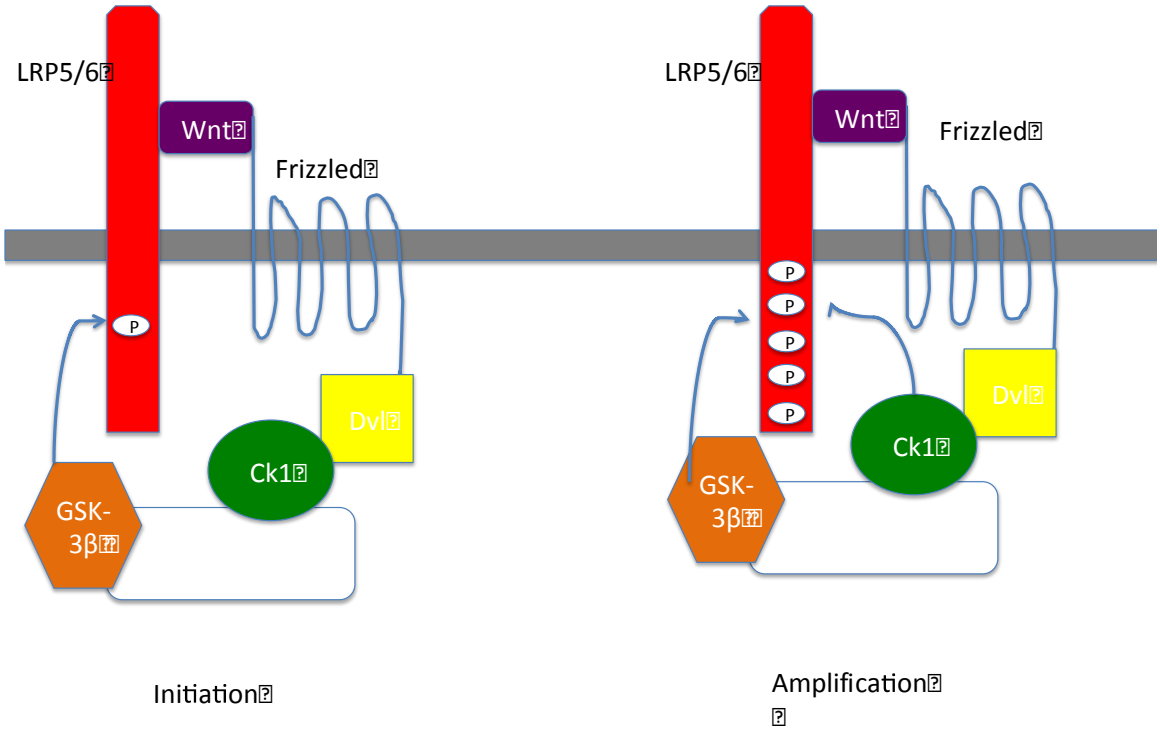

Figure 1: Fz/LRP5/6 complex stabilization. Binding of Wnt to Fz and LRP5/6 recruits Axin, which recruits GSK-3β and CK1. GSK-3β and CK1 phosphorylate all five PPPSPXS motifs on LRP5/6, which is essential for downstream signaling.

When Wnt is not bound to Fz and LRP5/6, β-catenin is degraded via the Axin complex, which is composed of the proteins Axin, GSK-3β, APC (**A**denomatous **P**olypsis **C**oli), and CK1. Axin has separate domains that interact with GSK-3β, CK1, and β-catenin, ultimately positioning the complex to be able to phosphorylate β-catenin. Once oriented correctly,

GSK-3β phosphorylates β-catenin on residues Thr 41, Ser 37, and Ser 33; CK1 will then phosphorylate β-catenin on residue Ser 45. Additionally, GSK-3β and CK1 will phosphorylate both Axin and APC, enhancing the association of the complex and therefore phosphorylation of β-catenin (Kimelman and Xu 2006). Phosphorylated β-catenin is then recognized by β-Tcrp, which ubiquitinates it, marking it for degradation by the proteasome (Figure 2).

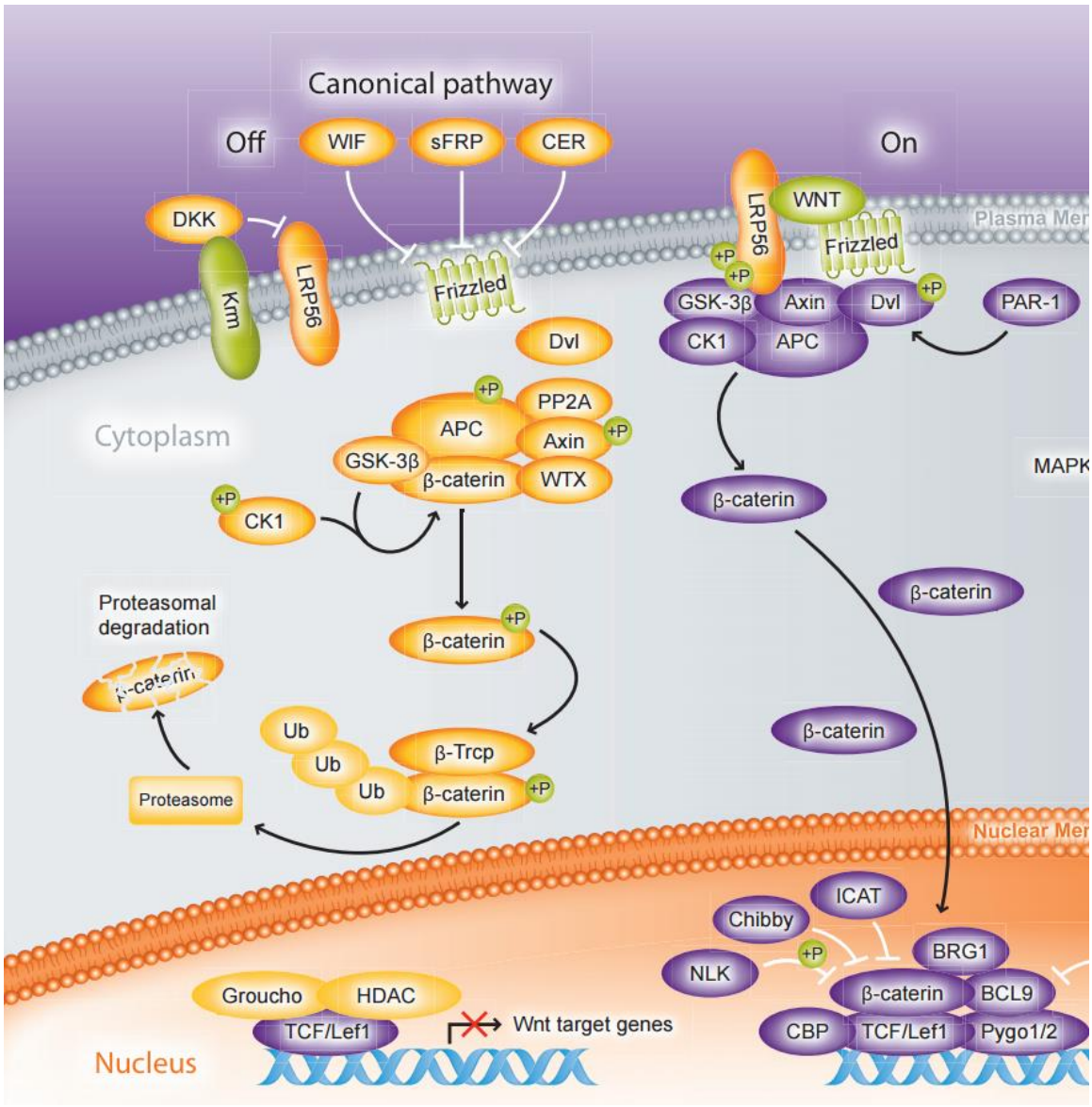

Figure 2: Wnt Signaling. In the absence of Wnt ligands, cytosolic β-catenin is continuously phosphorylated within the β-catenin destruction complex, which consists of APC, GSK3β, CK1 and Axin. Phosphorylated β-catenin is targeted for ubiquitin-dependent proteasomal degradation in the cytosol. Upon Wnt binding to to Fz and LRP6, β catenin accumulates in the cytosol and translocates into the nucleus and interacts with TCF/LEF1 for activation of Wnt-

responsive genes (http://docs.abcam.com/pdf/stemcells/Wnt-signaling-pathway.pdf, November 28, 2014).

Dkk1

The Dickkopf (Dkk) family is a group of glycoproteins that are known to interact with components of the Wnt pathway. The Dkks have two conserved cysteine rich domains, Cys-1 and 2. Structurally, the Dkks are similar to proteins in the colipase family, especially in the Cys-2 domain (Figure 3) (Krupnik et al. 1999). The homology between the Cys-2 domains of the two families suggests that the two families could function in a similar function. Indeed, colipases are known to interact with lipids, suggesting that Dkks could possibly use this lipid interaction to associate with the cell membrane, where Wnt proteins are known to associate. This relationship suggests a mechanism that facilitates Wnt protein-Dkk interaction (Aravind and Koonin 1998).

The four members of the Dkk family, Dkk1, 2, 3, and 4 are fairly evolutionarily conserved across most organisms (including humans), with the exception of *Caenorhabditis elegans* and *Drosophila melanogaster* (Nierhs 2006). Dkk1 inhibits the Wnt pathway by binding to LRP 5/6, which blocks the Wnt-Fz complex from binding LRP 5/6. Additionally, Dkk1 binding to LRP5/6 can induce endocytosis of LRP from the cell membrane (Cruciat and Niehrs 2013). Although disputed, there is evidence that it can also inhibit Wnt signaling by binding to the Kremen 1 and 2 cell surface receptors (Mao et al. 2002). Upon Dkk1 binding to both LRP and Kremen,

endocytosis of LRP from the cell membrane is induced (Kawano and Kypta 2003).

Figure 3: Conserved Cysteine domains in the Dkk family and their homology with the Colipase family.

DAX-1

DAX-1 (**D**osage sensitive sex reversal, **A**drenal hypoplasia congenita, critical region on the **X** chromosome, gene **1**) is an orphan nuclear hormone receptor encoded by the gene NR0B1. The Nuclear Hormone Receptor (NHR) superfamily is a group of transcription factors that act to enter the nucleus and change target gene expression. They can act in different ways, but are mostly active upon ligand binding, which is usually a hormone (Figure 4). However, certain NHRs, such as DAX-1, do not have a ligand, and are identified as orphan Nuclear Hormone Receptors (Aranda

and Pascual 2001).

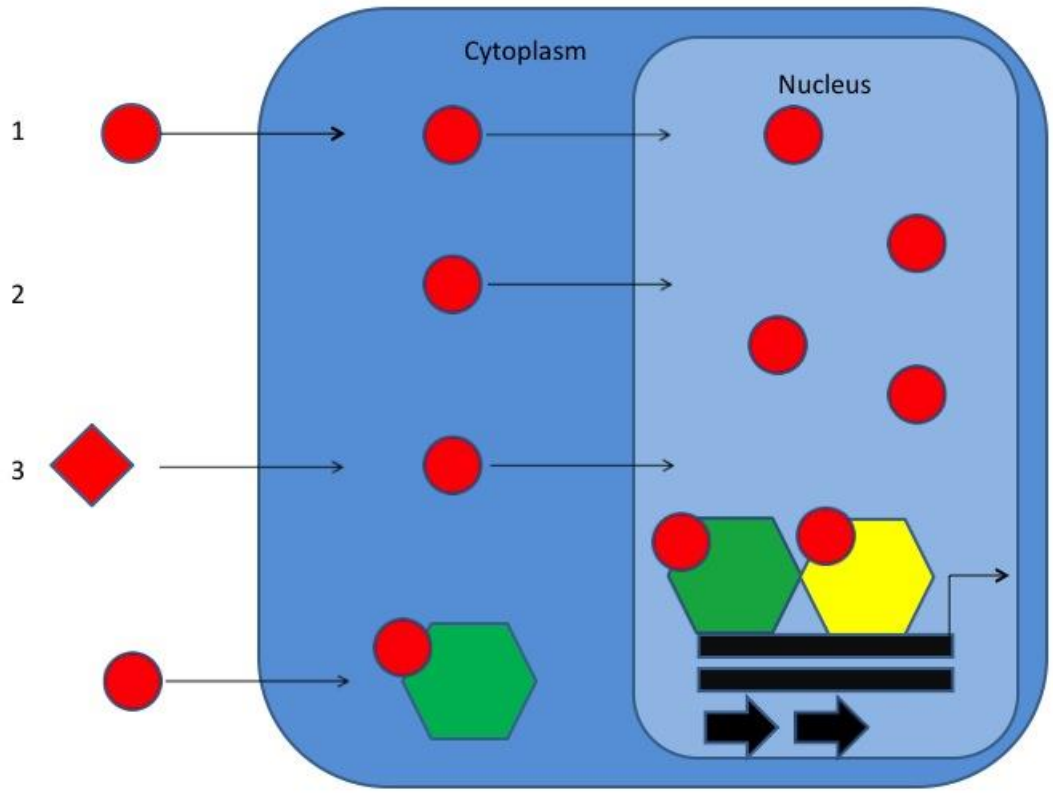

Figure 4: Mechanisms of activation of NHRs. 1. A ligand is synthesized in another cell and binds to the NHR in the cytoplasm. 2. A ligand is synthesized within the cell and binds to the NHR in the cytoplasm. 3. A ligand is metabolized in the cell and the metabolite binds the NHR in the cytoplasm. In all three cases, ligand binding allows the NHR to translocate to the nucleus. Once the NHR is in the nucleus, it will generally form a dimer or be part of a larger protein complex in order to change transcription of target genes.

Typical NHR structure starts with the N-terminal domain, which is adjacent to the DNA Binding Domain (DBD). The hinge region follows, which is located between the DBD and Ligand Binding Domain (LBD). The C-terminal domain is located next to the LBD and flanks the end of the protein (Figure 5).

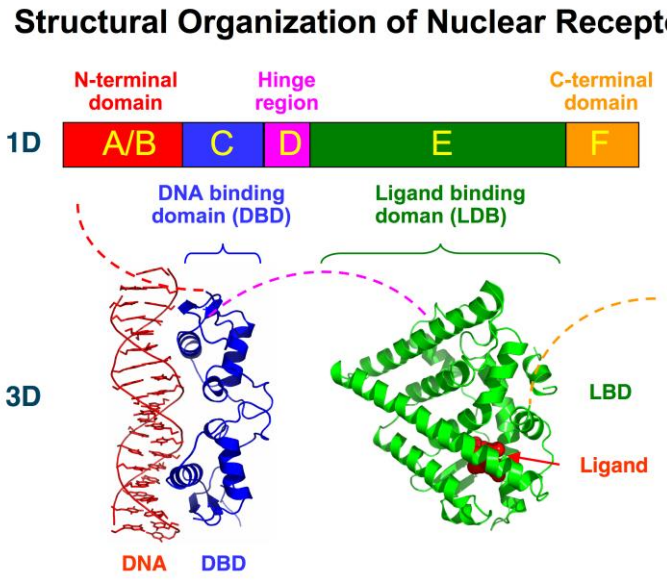

Figure 5: Typical NHR Structure. First, a linear depiction of NHR structure: the N-terminal domain (A/B), DBD (C), Hinge region (D), LBD (E), and C-terminal domain (F). Additionally, the three dimensional structure of an NHR's (Estrogen Receptor Alpha) DBD binding DNA and LBD binding ligand. **(**http://upload.wikimedia.org/wikipedia/commons/3/3e/Nuclear_Receptor_Structure.png, November 24, 2014)

The N-terminal domain of NHRs often contains a hypervariable AF-1 (**A**utonomous transcriptional activation **F**unction) domain that is responsible

for ligand independent activation of the NHR. The AF-1 domain allows NHRs to be promoter or cell-specific, which contributes to the NHR's specificity. Additionally, the N-terminal domain can be modified via phosphorylation, thereby modifying the function of the NHR (Aranda and Pascual 2001).

The DBD allows NHRs to recognize specific sequences of DNA. It has conserved Cys residues that form two zinc finger motifs, which allow the NHR to bind to the major groove of DNA. The hinge region of the NHR links the DBD and LBD and allows for rotation in the DBD. The hinge may contain nuclear localization signals, and possibly play a role in binding to other proteins (Aranda and Pascual 2001). Finally, the LBD is a multifunctional domain that is the site of ligand binding as well as mediation of dimerization and interaction with other proteins. It can also contain an AF-2 domain, which is similar to the AF-1 domain but functions in ligand-dependent transcriptional change (Wurtz et al. 1996).

DAX-1 is a unique NHR that has several key differences from the typical NHR. One difference is that DAX-1 has no known ligand, making it an orphan NHR. This could be due to the fact that the LBD and C-terminal domain are only partially conserved. Interestingly, even though it doesn't have a known ligand, it does have an AF-2 domain, which is dependent on a ligand to cause transcriptional change. It also carries a 26 amino acid long insertion in the LBD that is not found in other NHRs. Because this insertion is conserved in both mouse and humans, it could possibly play a

role in DAX-1 functionality (Lalli 2014). Another difference can be found in the DBD. DAX-1 lacks the typical zinc finger motifs of a NHR. Rather, it has a novel Cys-rich domain of about 70 amino acids, containing three LXXLL repeats, which are thought to have some function in the ability to regulate transcription (Ehrlund and Treuter 2012).

In early embryonic development, DAX-1 plays a key role in the development of the adrenal glands as well as gonads (Niakan and McCabe 2005). In males, the presence of the Y chromosome leads to expression of SRY (**S**ex-determining **R**egion **Y**), also known as TDF (**T**estes **D**etermining **F**actor). SRY activates the expression of SOX9 (**S**RY-related HMG b**ox9**) and inhibits the expression of DAX-1, which allows for the expression of SF (**S**teroidogenic **F**actor **1**). Together, SOX9 and SF1 will lead to the development of testes. In females, the absence of the Y chromosome, and therefore SRY, leads to the expression of DAX-1, inhibition of SOX9 and SF1, and the development of the ovaries (Figure 6).

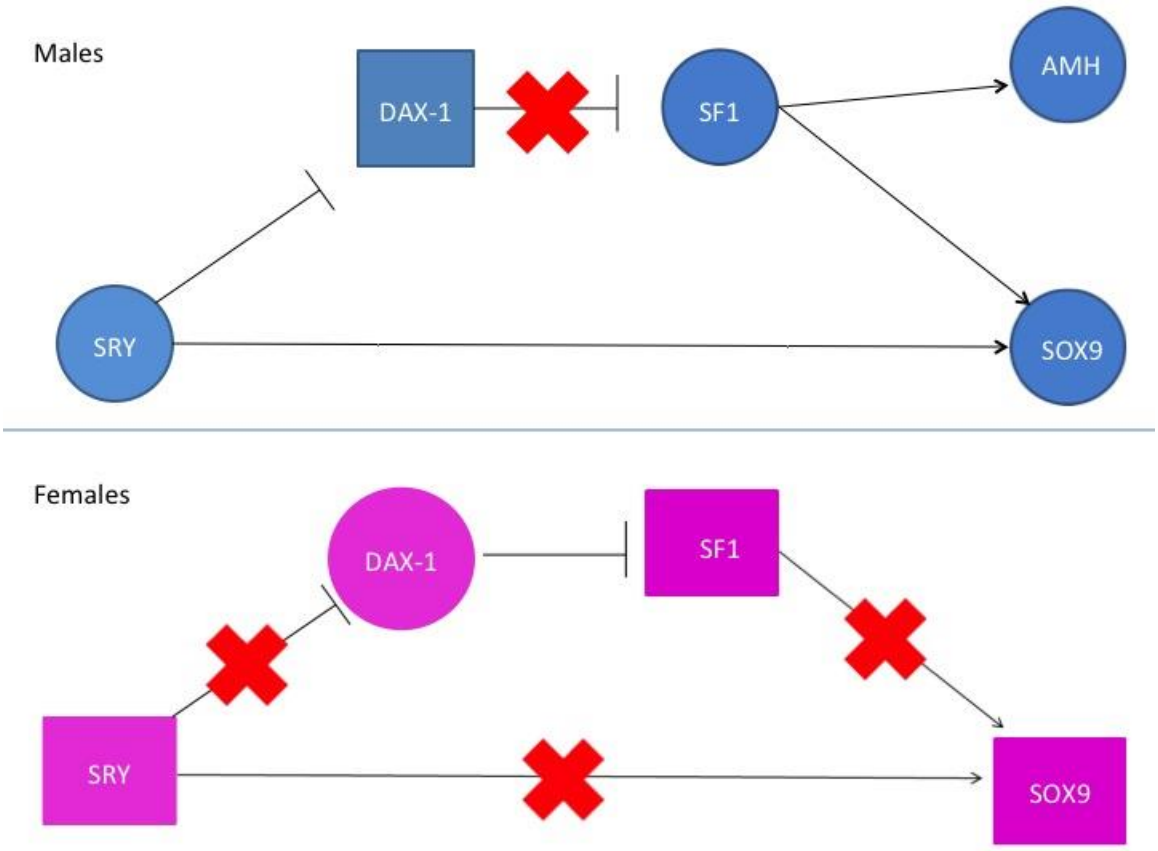

Figure 6: Interaction between SRY and DAX-1 in males versus females. In males, SRY will activate inhibit the expression of SOX9, while inhibiting the expression of DAX-1. These inhibitions allow SF1 to be expressed, which allow SF1 and SOX9 to differentiate primordial germ cells into the Leydig (Interstitial) and Sertoli (Sustentacular) cells of the testes. In females, there is no SRY present, allowing DAX-1 to inhibit the expression of SF1, which allows primordial germ cells to differentiate (by default) into the follicular, theca, and granulosa cells of the ovaries.

Given DAX-1's known role in the transcriptional regulation of genes in early embryonic development (Torres 2013), my thesis further investigates the transcriptional regulation of DAX-1 upon Dkk1. Specifically, I aim to demonstrate that DAX-1 is a negative regulator of Dkk1 during early embryonic development by comparing levels of gene expression between untreated mESC and mESC treated with siRNA to knockdown DAX-1. By artificially decreasing DAX expression via RNAi, I expect Dkk1 expression levels to increase.

Materials and methods

Cell Culture

mESC were cultured with a modified Dulbecco's Modified Eagle Medium (DMEM) in a 75mL flask that was pretreated with a 0.1% gelatin solution for at least one hour prior to passage. 15% fetal bovine serum, 1% non-essential amino acids, 1% penicillin/streptomycin, 50 µL leukemia inhibitory factor, 7 µL basal medium eagle, 12 mL L-glucose, and 10 mL sodium bicarbonate were added to the modified DMEM. Briefly, cells were first washed with 6 mL of Phosphate Buffered Saline (PBS) and then treated with 2 mL of trypsin. After five minutes of incubation, a sample of suspended cells was removed and pelleted at 1,000x g for five minutes. Cells were resuspended in 6 mL of DMEM and passaged in a 1:10 ratio.

DAX-1 Knockdown

DAX-1 gene expression was knocked down by RNAi as outlined by Alexandra Maramba (Maramba 2015).

RNA Isolation

Total RNA was collected from mESC after cell culture using the Qiagen RNA Easy miniprep kit. Briefly, before RNA collection, treated and untreated mESC were treated with trypsin and spun for five minutes at 1000x g. The centrifuged pellet was washed with 1X PBS and treated with 350 µL of Buffer RLT, disrupting

the pellet. The cells were vortexed for 10 seconds, and homogenized by passing cells through an 18 gauge needle attached to a syringe at least 10 times. 350 µL of 70% ethanol were added to the homogenized lysate and mixed by pipetting. 700 µL of the sample were transferred to an RNeasy spin column inside a 2 mL collection tube. The cell lysate was centrifuged at 8,000x g for 15 seconds. 700 µL of Buffer RW1 were added to the column after the flow-through was discarded. The column was again spun for 15 seconds at 8,000x g. After discarding the flow-through, 500 µL of Buffer RPE were added to the column and spun for 15 seconds at 8,000x g (for a total of two times). Finally, the column was placed in a new collection tube and 30 µL of water were added to the column and spun for one minute at 8,000x g to elute the RNA. RNA concentration was measured using a Nanodrop spectroscope.

cDNA Synthesis

cDNA was synthesized via the New England Biolabs First Strand cDNA Synthesis kit. First, 500 ng of RNA and two µL of random primer mix were added in an RNase free microfuge tube and heated to 70°C for five minutes in order to denature the RNA. Then, 10 µL of M-MuLV Reaction Mix and two µL of M-MuLV Enzyme mix were added to the tube. The tube was heated to 25°C for five minutes, followed by 42°C for one hour, ending with 80°C for five minutes. The cDNA was then diluted to 50 µL by adding 30 µL of PCR grade water.

Polymerase Chain Reaction and Gel Electrophoresis

Polymerase Chain Reaction (PCR) was performed in 20 µL reactions with 10 µL of Go Green Master Mix, 0.125 µL forward primer, 0.125 µL reverse primer, 7.75 µL PCR grade water, and two µL cDNA. All PCR reactions were performed as follows:

1. 95° five minutes
2. 95° 30 seconds
3. 58° 30 seconds
4. 72° 45 seconds
5. Repeat steps 2 through 4 30 times
6. 72° five minutes

After the PCR reaction was completed, the samples were electrophoresed at 110 Volts through a 2% agarose gel (in 1XTAE) containing ethidium bromide for 30 minutes and then visualized.

Table 1: Primers and Melting Temperatures used for PCR and qPCR of mESC cDNA.

Gene Name	Forward Primer (5'→3')	Reverse Primer (5'→3')	PCR Annealing Temp (°C)
DAX-1	TCC TGT ACC GCA GCT ATG TG	TCG AAG TGC AGG TGA TCT TG	57°C
Dkk1	GAG GGG AAA TTG AGG AAA GC	AGC CTT CTT GTC CTT TGG TG	57°C
GAPDH	ACA GCC GCA TCT TCT TGT GCA	GGG CTT GAC TGT GCC GTT GAA	57°C

Quantitative Polymerase Chain Reaction (qPCR)

qPCR was performed in 20µL reactions with 10 µL of iQ qPCR Master Mix (Bio-Rad), 0.2µL CXR reference dye, 0.250 µL forward primer, 0.250 µL reverse primer, 8.3 µL PCR grade water, and 1 µL cDNA. All qPCR reactions were performed as follows:

1. 95° five minutes
2. 95° 30 seconds
3. 58° 30 seconds
4. 72° 45 seconds
5. Repeat 2-4 30 times
6. 72° five minutes

Quantitative PCR was used to compare the gene expression of both Dkk1 and DAX-1 in untreated and RNAi DAX-1 knockdown mESC samples. GAPDH was used as a control. Each qPCR reaction was run in triplicate. Gene expression was measured via change in Cycle Threshold values (ΔΔCt). Ct values are measured by the amount of cycles in a qPCR reaction are necessary to reach a threshold value of fluorescence, which correlates to the amount of gene product existing in the reaction. ΔCt values are then calculated by subtracting the Ct values of the control (GAPDH) from target genes (Dkk1, DAX-1). ΔΔCt values are then calculated from this (2^-ΔCt).

Results

DAX-1 is known to play a role in maintaining the pluripotent state of mESC. However, a thorough examination of essential genes regulated by Dax-1 expression has not been elucidated. As part of her thesis research in the Tzagarakis-Foster lab, Alexandra Maramba utilized an siRNA mediated gene knockdown method to significantly reduce the amount of DAX-1 expression in mESC. I confirmed DAX-1 knockdown via Western Blot (Figure 7) and then used this method to measure the levels of gene expression of Dkk1, and how levels changed with and without the presence of DAX-1 in mESC. To compare levels of gene expression, I calculated ΔΔCt values from qPCR data.

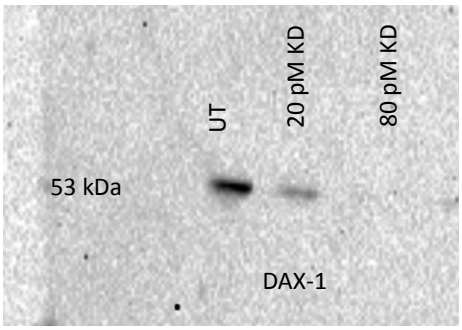

Figure 7: Confirmation of DAX-1 knockdown via Western Blot.

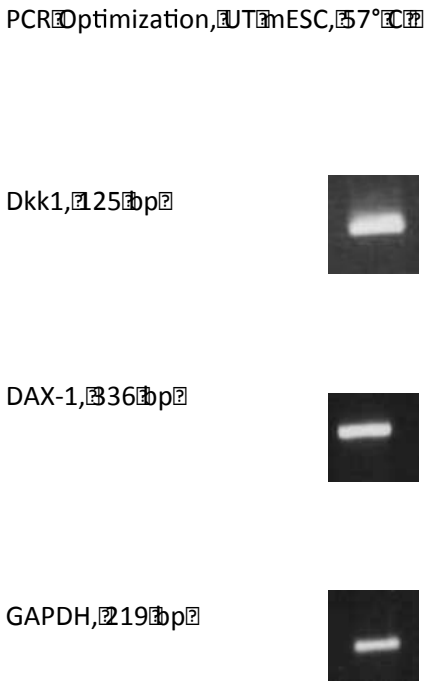

Figure 8: PCR Optimization of Melting Temperatures. Results of PCR amplification of Dkk1, DAX-1, and GAPDH at 57°C visualized on 1.5% agarose gel.

First, qPCR conditions were optimized via PCR. Melting temperatures for DAX-1 and Dkk1 as well as the control (GAPDH) were determined to be 57°C (Figure 8). After optimization, qPCR was performed. As seen in figure 9, ΔΔCt values for DAX-1 exhibit more than a twofold decrease from untreated cells to the mESC treated with 80pM DAX-1 siRNA. These values range from a ΔΔCt value of 10.03 in the untreated cells, to 4.33 for the 20pM DAX-1 knockdown, to 3.76 for the 80pM knockdown. Dkk1 ΔΔCt values exhibited more than a twofold increase when comparing untreated cells to the cells that had a DAX-1

knockdown. These values ranged from 0.41 in the untreated cells, to 0.85 in 20 pM knockdown cells, to .099 in the 80 pM knockdown cells (Figure 10).

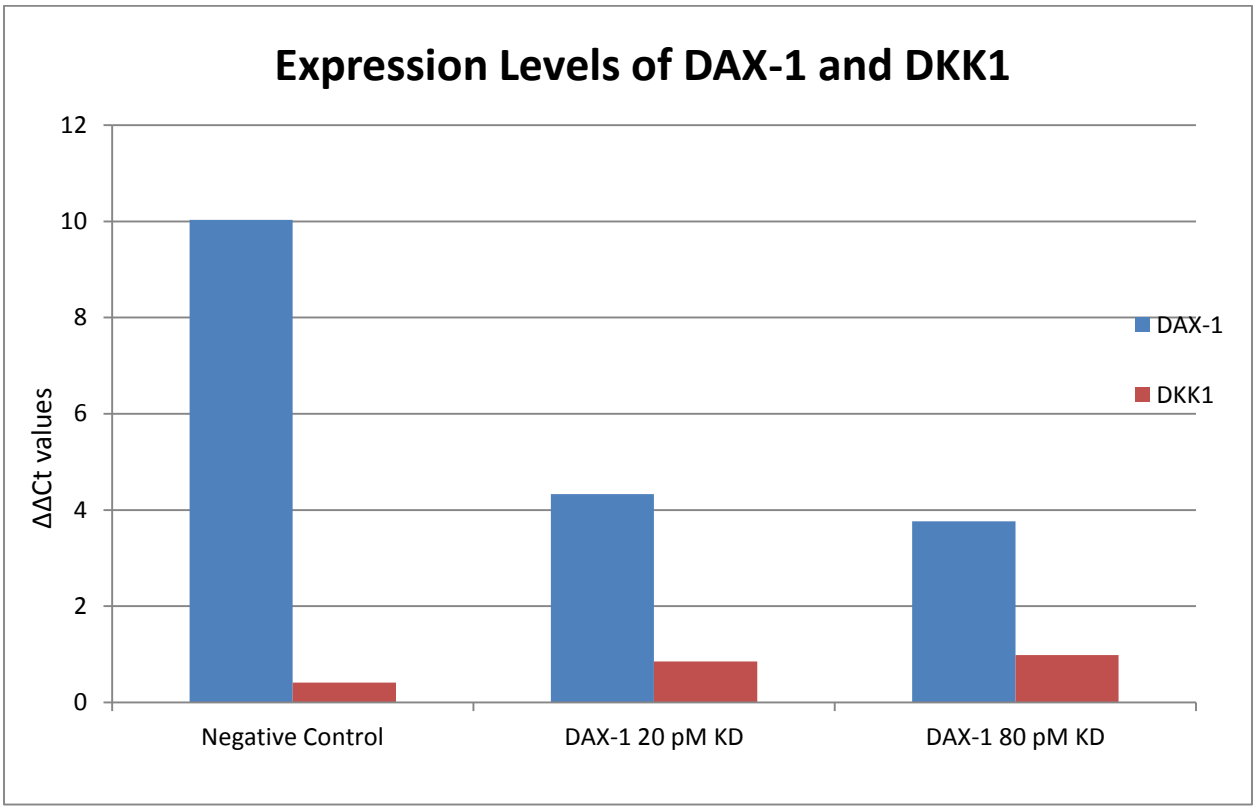

Figure 9: Comparison of ΔΔCt Values of DAX-1 and Dkk1. A graphical comparison of ΔΔCt values of DAX-1 and Dkk1 in untreated (NC) and DAX-1 knockdown mESC.

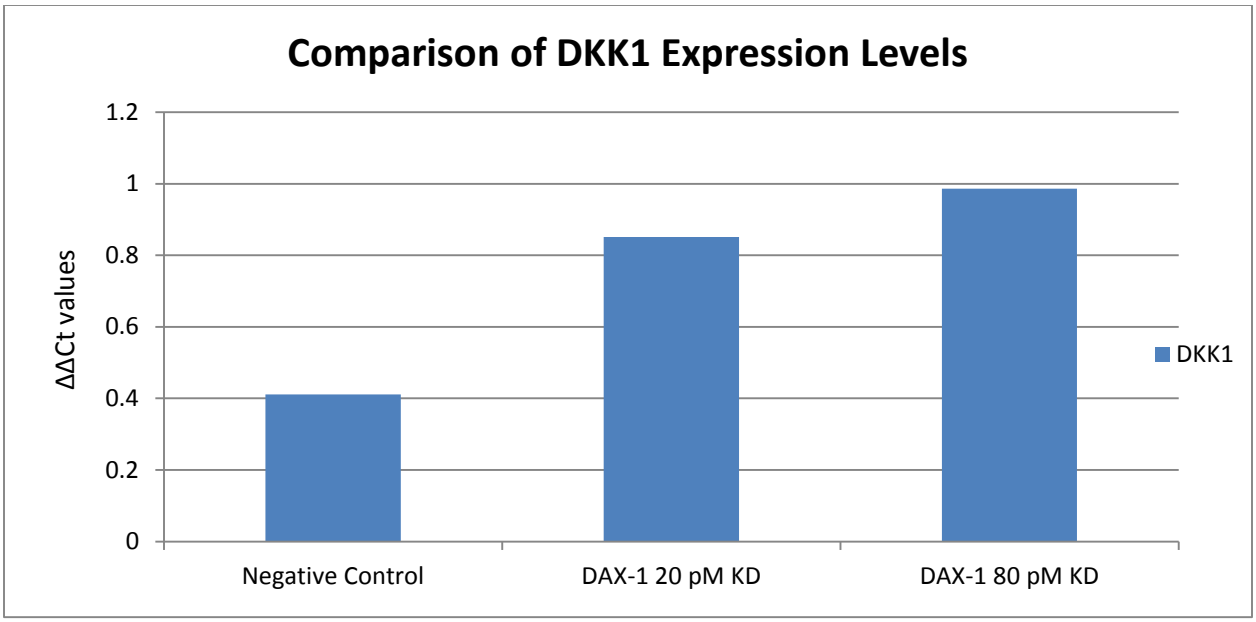

Figure 10: ΔΔCt Values of Dkk1. A graphical representation showing the increase in ΔΔCt values of Dkk1 from untreated mESC to DAX-1 knockdown mESC.

Discussion

Although Wnt signaling is one of the major signaling pathways in areas of biology such as development and cancer biology, little is known about one of its inhibitors, Dkk1. In this Honors Thesis Research Project, I investigated the relationship between Dkk1 and DAX-1. The results of the qPCR data agree with my hypothesis that DAX-1 is a negative regulator of Dkk1 gene expression. This agreement is supported by the over twofold increase in gene expression of Dkk1 when DAX-1 is knocked down. Additionally, another interesting fact revealed by the qPCR data is that the overall expression level of Dkk1 is very low in mESC whether or not DAX-1 is expressed in the cell. Since DAX-1 is an important player in maintaining pluripotency, it is expected that the level of DAX-1 expression should be relatively high. However, in untreated cells, the gene expression levels of DAX-1 are 50 times that of Dkk1. Although further research is necessary, these data suggest that Dkk1 could be a player in the loss of pluripotency. In fact, part of DAX-1's major role in pluripotency could be to make sure Dkk1 expression is kept low.

There are a few possible extensions of this research project. First, one could quantify the protein expression of both DAX-1 and Dkk1 via western blot to compare with the results of the qPCR. I would expect these levels of expression to agree with one another, and find that in untreated cells, there is a large amount of DAX-1 and little or no Dkk1. In cells with DAX-1 knocked down, I would expect to find little or no DAX-1 protein product while having a slight

increase in the amount of Dkk1. Most likely, one would have to perform an immunoprecipitation in order to achieve significant protein data.

Another extension of this project is to perform a CHIP (**Ch**romatin **I**mmuno**p**recipitation) Assay in order to discover what proteins, along with DAX-1, are binding to the promoter of Dkk1 to decrease its gene expression. It is highly unlikely that DAX-1 binds by itself to the promoter since it usually acts as part of a greater protein complex. Several proteins significant in the maintenance of pluripotency have already been shown to bind either to the Dkk1 promoter in primary literature such as β-catenin, TCF, Nanog, Oct4, Pou5f1, Otx2, and MesP. The CHIP assay would be used to investigate whether or not these proteins do bind with DAX-1 to the Dkk1 promoter.

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